T-Cell Nonmalignant Clonal Proliferation in Ataxia Telangiectasia: A Cytological, Immunological, and Molecular Characterization

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Cytogenetically abnormal T-cell nonmalignant clones are a characteristic feature of ataxia telangiectasia (AT). Here, we study a t(14;14) clone from a patient with AT, and provide a cytological, immunological, and molecular characterization. This cellular population is clonal at the molecular level, but is phenotypically heterogeneous, with CD4^+CD8^- and CD4^-CD8^+ cells. Although these cells do not divide in the peripheral blood, a majority of them are found in G1 phase and express the membrane antigen 4F2, a very early marker of activation. Many similarities are found between this nonmalignant AT clone and T-cell prolymphocytic leukemia at the morphologic, cytogenetic, and immunologic levels, despite the different clinical courses associated with these proliferations. We hypothesize that the t(14;14) translocation is linked to the abnormal morphology and immunophenotype of the AT clone cells, but that this translocation confers only a preactivated state to the cells. A complete malignant transformation would then be due to secondary events.


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MATERIALS AND METHODS

Patient and cells. Blood samples were obtained from a 13-year-old boy with typical AT. An AT clone with t(14;14) rearrangement involving approximately 70% of the metaphases has been observed since 1982 and was previously reported. The last WBC count was 8.9 x 10^3/L with 55% granulocytes, 36% lymphocytes, and 9% monocytes. Blood smears were stained by the May-Grunwald-Giemsa stain. Peripheral blood lymphocytes were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density sedimentation. In some assays, T lymphocytes were purified by neuraminidase-treated sheep RBCs rosetting. Cells from control individuals were isolated with the same procedures.

Monoclonal antibodies. Monoclonal antibodies (MoAbs) conjugated to fluorescein isothiocyanate (FITC) and to phycoerythrin (PE) were anti-CD4 (IOT4), anti-CD8 (IOT8), (Immunotech, Luminy, France) and anti-CD3 (OKT3) (Ortho Diagnostics, France). Unconjugated MoAbs used for indirect staining were anti-CD7 (IOT7) (Immunotech), anti-CD1 (OKT6), anti-CD9 (OKT9) (Ortho), anti-HLA-DR (D1-12), anti-CD25 (anti-Tac), WT31 (recognizing a framework part of the TCRA-TCRB complex), and 4F2. Immunofluorescence staining. For the indirect staining procedure, incubations and washing were carried out at 4°C with an appropriate dilution of the MoAb. Cells were washed twice and incubated for 20 minutes with FITC-conjugated goat anti-mouse antibody (Nordic Immunology, Netherlands). Cells were then analyzed after two additional washes. For direct staining procedure, the second step was omitted. Cells from normal individuals were used as controls.

Flow cytometric analysis and cell sorting. Flow cytometric analysis and cell sorting were performed on a FACSTAR (Becton Dickinson, Mountain View, CA) flow cytometer equipped with a 5 W argon ion laser (Coherent Inc, Palo Alto, CA). The fluorescence was excited by the 488 nm beam of the laser at 200 mW. Green and red fluorochromes were detected by photomultipliers fitted with 540 nm and 590 nm bandpass interference filters (Oriel, France), respectively. The acidine orange fluorescence was excited under the same condition. The green (DNA content) and the red (RNA content) fluorochromes were detected by photomultipliers fitted with 530 nm bandpass and 620 nm longpass filters, respectively. For each analysis, data from 10,000 cells were collected and processed with the Consort-30 computer program (Becton Dickinson) on a Hewlett-Packard 300 desktop computer.

Karyotype and cell culture methods. After cell sorting, cells were washed and resuspended in RPMI 1640 (GIBCO, Paisley, UK) supplemented with glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (0.1 mg/mL), PHA (15 μg/mL), DMSO (Difco, Detroit), human serum AB (10%) and 5 x 10^3/mL irradiated (4,500 rad).
allogenic mononuclear cells. Crude T-cell growth factor was prepared as previously described and added in the medium at 10% final. Cells were grown for 72 hours and colchicine (Colchineo, Houde, France) (0.04 μg/mL) was added two hours before harvesting. R-banded karyotype was performed according to our standard technique. DNA extraction and Southern blot analysis. High molecular weight DNA was extracted from different sorted cells and from mononuclear cells of the AT patient, depleted of T lymphocytes by rosetting. DNA was then digested to completion by HindIII restriction enzyme (Genofit, France), separated by electrophoresis on a 0.7% agarose gel, and transferred to nylon membrane (Zetabind; Cuno, Meriden, Conn) using sodium hydroxide 0.4 N as transfer solvent. The genomic blot was hybridized to a 32P-labeled TCRB probe radiolabeled by the random priming method. The blot was washed to a final wash in 0.015 mol/L NaCl, 0.0015 mol/L of trisodium citrate, and 0.1% sodium dodecyl sulfate at 65°C and autoradiographed for one to eight days at -70°C with an intensifying screen. The TCRB probe was the 770 bp PsiI fragment of the Jurkatt TCRB cDNA. Cell cycle analysis. Acridine orange cell cycle analysis was performed according to Darzynkiewicz et al. Briefly, 2 x 10⁷ cells in 200 μL of RPMI 1640 were permeabilized by adding 400 μL of a solution containing 0.1% Triton X-100, 0.08 mol/L HCl, and 0.15 mol/L NaCl. Then, 1.2 mL of a solution containing acridine orange 25 μmol/L (Polysciences Inc, Warrington, PA), EDTA 10⁻³ mol/L, sodium phosphate 0.2 mol/L and citric acid 0.1 mol/L, pH 6, was added five minutes before the analysis. The analysis of T lymphocytes from a healthy donor was used to identify the G₀ and G₁ cell cycle phases.

RESULTS

Cell morphology. Examination of the blood smears after standard staining revealed two types of abnormalities. One abnormal population of lymphocytes (32% of the lymphocytes) was characterized by a small size, a high nucleocytoplasmic ratio, a basophilic cytoplasm devoid of granules, a well condensed chromatin, and a prominent unique nucleolus (Fig 1A) and sometimes by a nucleus displaying multiple clefts (Fig 1B). Another abnormal population (28% of the lymphocytes) was characterized by an abundant and less basophilic cytoplasm and an irregularly shaped nucleus with moderately condensed chromatin and a prominent nucleolus (Fig 1C). However, some abnormal lymphocytes could not be classified within the preceding categories because of an intermediate morphology.

Immunophenotyping analyses. The complete immunophenotype is summarized in Table 1. The dual color flow cytometric analysis using anti-CD4 and anti-CD8 MoAbs is presented in Fig 2. One third of the cells had a CD4⁺CD8⁺ phenotype and a half had a normal CD8⁺ phenotype. However, no clear-cut separation was observed between these populations but rather a continuous gradient of the CD4 antigen density in a large CD8⁺ population. The minor CD4⁺CD8⁺ population was clearly separated from the two others. A dual color analysis performed with CD3 and WT31 MoAbs showed no dissociation between the two antigens (data not shown). The cells did not express the CD1 antigen but expressed CD3, WT31, CD7, and CD2 antigens. Among the activation-related antigens, only the 4F2 antigen was expressed, while CD25 (Tac), CD9, and HLA-DR antigens were absent.

Cell cycle analysis. A cell cycle analysis was performed on T lymphocytes by an acridine orange assay. The result is presented in Fig 3. A diploid DNA content was found in almost all the cells (99.5%), indicating that almost no cell was in S/G₂-M phases. Nevertheless, given their high RNA content, a major population was found in the G₁ phase, confirming the presence of activated lymphocytes in the peripheral blood.

Fig 1. Examples of small (A, B) and large (C) abnormal lymphocytes (May-Grunwald-Giemsa staining).
Characterization of the Different Subpopulations

Cytogenetic analyses. We investigated the presence of the AT clone cells among the different subpopulations. For this purpose, 1.5 × 10^6 T lymphocytes were sorted according to their phenotype. Cells were immediately grown in the conditions described above. Results are summarized in Table 2. A large majority of the metaphases (82%) from the CD4^+CD8^+ cell culture and 32% of those from the CD4^-CD8^- cell culture bore the t(14;14) translocation, whereas only 5% of the metaphases from the CD4^-CD8^- cell culture bore the translocation. Other chromosome rearrangements, such as chromosome 7 inversions, were found in the three cultures but at a much higher rate in the CD4^-CD8^- culture. These sporadic rearrangements were never found in the clonal t(14;14) cells.

Southern blot analysis. Rearrangements of the TCRB genes were searched for by Southern blot analysis. Results are presented in Fig 4. Two nongermline bands were revealed in the DNA from the CD4^-CD8^- cells after HindIII digestion, whereas the 8 and 3.5 kb germline bands, corresponding to the Cβ2 and Cβ1 constant regions of the TCRB, respectively, were not detected. The 6.5 kb band is constant and corresponds to the 3' fragment of Cβ2. Identical nongermline bands were detected in the HindIII digested DNA from the CD4^-CD8^- cells, indicating the presence of the same clonal population. However, the presence of faint 3.5 and 8 kb bands indicates the presence of a significant proportion of nonclonal cells. Given the small blood sample available from the patient and the low frequency of the CD4^-CD8^- population, sufficient DNA from this population could not be obtained.

DISCUSSION

In the present study, we provide new data on an AT clone with a t(14;14) translocation. This t(14;14) AT clone was observed for the first time in 1982. Although there is no evidence for a malignant process, we cannot exclude such possibility. After more than 5 years of regular follow-up of the AT patient, no change in the clinical status was observed except neurological impairment. WBC counts remained stable and cytogenetic analyses revealed only a small increase in the frequency of translocated cells. Such an evolution is common to a majority of the nonmalignant AT clones.3 High resolution R-banding karyotypes and in situ chromosomal hybridization data have already been published on this clone.4,5,19

In this patient, a majority of the peripheral lymphocytes was found to be abnormal. A first abnormal population is composed of small lymphocytes morphologically indistinguishable from T-cell prolymphocytic leukemic (T-PLL) cells. The second abnormal population is composed of large and less mature atypical lymphocytes.

Immunophenotypic studies of the peripheral blood cells also revealed the existence of an abnormal population with a CD4^+CD8^- phenotype. After purification of these cells by cell sorting, we demonstrated that this population was largely constituted by t(14;14) AT clone cells. In this CD4^+CD8^- population, rearrangements of the two TCRB genes were detected by Southern analysis, confirming that this population was clonal. By the same means, both cytogenetic and molecular data revealed the presence of AT clone cells in the CD4^-CD8^- population. About one third of the metaphases obtained from this population bore the t(14;14) translocation. The Southern analysis confirmed that some of the CD4^-CD8^- cells had the same clonal rearrangements as the CD4^-CD8^- cells. It is thus likely that one population derived from the other. However, because no clear-cut separation existed between the CD4 positive and negative cells among the CD8^-, the clonal cells probably have a continuous range of CD4 antigen from negative or very low expression to high expression. Karyotypic data revealed almost no t(14;14) cells in the CD4^-CD8^- population, showing that the AT clone was confined to the CD8^- population.

A dramatic increase of sporadic inversions and translocations is characteristic of AT.2 Such chromosome rearrangements were found in the cultures from the three phenotypi-
Table 2. Short-Term Culture Karyotypes From Sorted Populations

<table>
<thead>
<tr>
<th>Sorted Cell Phenotypes</th>
<th>No. of t(14;14) Cells (%)</th>
<th>No. of Cells Without t(14;14)</th>
<th>Rearrangements in Cells Without t(14;14)</th>
<th>No. of Analyzed Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD8+</td>
<td>136 (85)</td>
<td>29</td>
<td>6*</td>
<td>165</td>
</tr>
<tr>
<td>CD4+CD8-</td>
<td>19 (32)</td>
<td>36</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>CD4-CD8+</td>
<td>5 (5)</td>
<td>92</td>
<td>1†</td>
<td>97</td>
</tr>
</tbody>
</table>

For karyotype analyses, 10^6, 10^8, and 2 x 10^8 sorted cells from the CD4+CD8+, CD4+CD8-, and CD4+CD8- populations, respectively, were grown as described in the text.

*One of the six cells had two inv(7) and no normal chromosome 7.
†Two cells with t(7;14).
‡One cell with inv(14).

cally different populations. Surprisingly, inversion of chromosome 7, which is the most frequent sporadic rearrangement in AT, occurred at a much higher frequency in the nonclonal CD4+CD8+ cells. The significance of this observation and its relevance to the abnormal thymic development in AT are under investigation.

In a previous study, we demonstrated that one of the TCRA genes of this AT clone is split by the t(14;14) translocation. An abnormal or absent TCRA chain expression could then be suspected. A normal T-cell receptor complex is formed by the TCRA and TCRB chains and the CD3 complex, detected by WT31 and anti-CD3 MoAbs, respectively. As WT31 expression was found on most of the T cells without dissociation from CD3 expression, we suppose that a normal TCRA chain was coded by the TCRA gene not involved in the translocation.

The 4F2 antigen was expressed on approximately 70% of the lymphocytes. This antigen appears on the T lymphocyte membrane as early as two hours after lectin stimulation, long before the expression of the transferrin receptor (CD9), the interleukin-2 receptor (CD25), and the HLA class II molecules. These latter antigens—CD9, CD25, and HLA class II—were not expressed at a significant level on the AT patient lymphocytes. Therefore, the 4F2+CD9-CD25-HLA- lymphocytes that are presumed to be the t(14;14) clonal population, have a phenotype identical to early activated lympho-
cytes. Furthermore, the same proportion of cells were found in a G1 state in an acridine orange assay. Although we cannot exclude that this unusual phenotype may be related to the immature CD4+CD8+ cells, these data support the possibility that the t(14;14) clonal cells are in a stable and early state of activation, but do not proliferate in the peripheral blood. This activation state remains to be defined at the molecular level.

The absence of CD1 antigen, and the presence of a normal level of CD3-WT31 complex and of both CD4 and CD8 antigens is a phenotype rarely found either in normal or in diseased individuals. Such populations are present as a very minor population in the peripheral blood from normal individuals21 and from AT patients without detectable AT clone (unpublished data). In T-cell malignancies, the unusual CD3+CD4+CD8+ phenotype has been reported very rarely.22-24 By contrast, two of three phenotyped T-cell leukemias in AT had such a phenotype.8,9,25 The third case had a CD4+CD8+ phenotype.26 In nonmalignant AT clones, in addition to the case reported herein, one case was again the CD4+CD8+ phenotype. Thus, nonmalignant clones and leukemias occurring in AT seem to arise from the same CD1-CD3+CD4+CD8+ or CD1-CD3+CD4-CD8+ populations. In a few cases, both phenotypes coexisted.25

Striking similarities are found between nonmalignant AT clones and T-PLL, despite the different clinical courses associated with these proliferations. First, the morphologic aspect of the abnormal cells found in this t(14;14) AT clone is indistinguishable from the T-PLL morphology.27 Second, a CD3+CD4+CD8+ phenotype similar to that of this AT clone is found in about 20% of the T-PLL.28-29 Third, rearrangements of the chromosome 14, which juxtaposed bands 14q11.2 and 14q32.1, i.e., inv(14) and t(14;14), are particularly frequent in T-PLL as they occur in two thirds of the cases30 and occur almost constantly in nonmalignant AT clones.31-33

In summary, the t(14;14) AT clone cells were found to be a major peripheral T-cell population with both CD4+CD8+ and CD4-CD8+ phenotypes. Clonality was confirmed by analysis of the TCRB rearrangements. Approximately the same proportion of peripheral lymphocytes that we believed to be the AT clone cells, were identified by their abnormal cell morphologies, their expression of the 4F2 antigen and by their G1 phase state. We and others previously hypothesized that a gene located to the 14q32.1 band is disregulated by the vicinity of the constant region of the TCRA gene due to the chromosome 14 rearrangement.9,33-36 This disregulation could then lead to the cellular abnormalities observed in this t(14;14) AT clone. Because such translocations are also observed in T-PLL, we suppose that secondary events are necessary for a malignant transformation. Such events could be the deletions of one chromosome 20, which are found in four of five cases of malignant AT leukemias,4,10,23 or the multimysisms of the long arm of chromosome 8, which are found in two thirds of the T-PLL.24

ACKNOWLEDGMENT

The authors wish to thank T.W. Mak for the gift of the TCRB probe, D. Charron, T.A. Waldmann, J.E. de Vries, and A. Bernard for the gift of D1-12, Tac, WT31, and 4F2 monoclonal antibodies, respectively. We also thank G. Flandid for reviewing the cellular morphology, G. Bismuth for his help in the acridine orange assay, and Gena Parris for preparing the manuscript.

NOTE ADDED IN PROOF

Since the submission of this manuscript we have studied six other AT clones. Five of them, bearing an inv14 or a t(14;14), showed cellular morphologies similar to the case described above, whereas a t(X;14) AT clone was morphologically normal.

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